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LABORATORY STUDY

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Diacylglycerol kinase epsilon protects against renal ischemia/reperfusion injury in mice through Krüppel-like factor 15/klotho pathway

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ABSTRACT

Although recent studies have indicated that mutations in the gene encoding diacylglycerol kinase epsilon (DGKE) result in some proteinuria related hereditary kidney diseases, the DGKE expression pattern in the kidney and its contribution to acute kidney injury (AKI) remain unknown. Therefore, the present study was designed to detect the role of DGKE in mice with AKI. DGKE expression was time-dependently altered in the kidneys of mice with renal ischemia/reperfusion injury (IRI). Compared with wild-type (WT) mice, DGKE- overexpressing mice (*Rosa26-Dgke*$^{+/+}$) exhibited protective effects against renal IRI, including reduced serum creatinine, blood urea concentration, tubular cell death and inflammatory responses as well as improved morphological injuries. Consistently, *in vitro*, DGKE overexpression in human renal proximal tubule (HK-2) cells also protected against oxygen-glucose deprivation (OGD)/reoxygenation-induced cell death. Mechanistically, DGKE regulated Klotho expression, at least partly *via* the transcription factor Krüppel-like factor (KLF) 15. Moreover, a significant reduction in DGKE was also found in kidneys from patients with ischemia-associated acute tubular necrosis (ATN). Collectively, our studies demonstrate that DGKE protects against AKI in mice at least partly through KLF15/Klotho signaling pathway, indicating that DGKE may present an innovative therapeutic strategy for treating patients with AKI.

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Introduction

Acute kidney injury (AKI) is a devastating clinical syndrome with high morbidity and mortality worldwide [1]. Renal ischemia/reperfusion injury (IRI), which is associated with transplantation, cardiac bypass surgery and shock, is a major risk factor for developing AKI and subsequent chronic kidney disease (CKD) [2]. Although substantial progress has been made in understanding the pathophysiology of AKI, the molecular mechanisms contributing to AKI remain unclear. Moreover, there is currently no satisfactory clinical treatment available to prevent or treat ischemic AKI [3,4]. Therefore, identifying the key and universal molecules involved in AKI may provide clues to develop new therapeutic strategies for patients with AKI.

Diacylglycerol kinases (DGKs) are intracellular lipid kinases that catalyze a reaction for the conversion of diacylglycerol (DAG) to phosphatidic acid (PA). Therefore, DGKs regulate various signaling transductions by terminating DAG signaling and activating PA-mediated pathways [5]. Currently, ten isoforms of mammalian DGKs have been identified and grouped into five types based on the homology of their structural features [6]. DGKE (DGKε, DGK5) is the only member of subtype III isozymes in the DGK family, which possesses several unique features, such as constitutively activity, the smallest molecule and a marked selectivity for arachidonic acid-containing DAG (AADAG) [7]. Emerging evidence has indicated that loss-of-function DGKE mutations cause a group of rare renal diseases including atypical hemolytic uremic syndrome (aHUS) [8] and membranoproliferative glomerulonephritis (MPGN)-like glomerular microangiopathy [9], which are also termed DGKE nephropathy [10]. However, the current understanding of DGKE biological functions in the kidney is very limited. DGKE is expressed in podocytes and endothelial cells [11], but whether it is present in other renal parenchymal cells, such as renal tubular cells, remains unknown. Given that in addition to intravascular hemolysis and thrombocytopenia, AKI is also

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one of the prominent clinical manifestations of aHUS [12], it is necessary to elucidate the function of DGKE in AKI. In this study, we found that the DGKE expression levels were significantly reduced in the kidney from biopsy-proven acute tubular necrosis (ATN) patients related to renal ischemia. We further found that DGKE-overexpressing mice (*Rosa26-Dgke*$^{+/+}$) exhibited protective effects against renal IRI. Mechanistically, DGKE protects against AKI in mice, at least in part through KLF15/Klotho pathway, suggesting that DGKE may represent an innovative therapeutic strategy for treating patients with AKI.

Materials and methods

Human renal biopsy samples

Renal biopsies were performed as part of routine clinical diagnostic investigation. Sections with a pathological diagnosis of ATN accompanied by ischemic pathological manifestations or from patients after renal transplantation who had experienced ischemia and reperfusion processes [13] were obtained from Department of Pathology, School of Basic Medical Sciences, Shandong University. Normal control samples were obtained from the healthy kidney poles of individuals who underwent tumor nephrectomies without renal diseases. The investigations were conducted in accordance with the principles of the Declaration of Helsinki and were approved by the Research Ethics Committee of School of Basic Medical Sciences, Shandong University after informed consent was obtained from the patients (Document No. ECSBMSSDU2018-1-050).

Mouse models of acute kidney injury

DGKE overexpressing mice (*Rosa26-Dgke*$^{+/+}$) were generated by Shanghai Southern Model Biotechnology Development Co., Ltd. (Shanghai, China). Twelve-week-old (20–25 g body weight) male *Rosa26-Dgke*$^{+/+}$ mice and age-matched wild-type (WT) mice were subjected to renal ischemia/reperfusion injury (IRI) as described previously [14]. In brief, mice were anesthetized with intraperitoneal pentobarbital (30 mg/kg body weight) after an overnight fasting (with free access to water). *via* a midline abdominal incision, bilateral renal pedicles were gently separated and clipped for 30 min using silvery arteriole clips (Fine Science Tools, Inc., Heidelberg, Germany). At the end of the ischemic period, the vascular clips were removed, and the kidneys were observed for 5 min to confirm reperfusion. The incision was then closed, and the animal was allowed to free access to food and water after recovery. During the anesthesia, rectal temperature was maintained at 37 °C with a temperature-controlled heating system (Themostar, RWD Life Science Co., Ltd, Shenzhen, China). Mice in the sham-operated (Sham) group were subjected to the same procedure except for clamping of the pedicles. At the end of reperfusion, blood samples were collected for renal function assessment. Anesthetized mice were then perfused with phosphate buffered saline at 150–160 mmHg *via* a butterfly 23 G needle inserted into the left ventricle to totally remove blood. Half of the kidney was harvested for Western blot and transcriptional analysis and the other part was further fixed in 4% paraformaldehyde for preparation of tissue sections. All experimental protocols for animal studies were approved by the Institutional Animal Care and Use Committee of School of Basic Medical Sciences, Shandong University (Document No. ECSBMSSDU2018-2-088) and conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Renal function and histology detection

Renal function was assessed by measuring blood urea nitrogen (BUN) and creatinine in serum *via* using an automatic biochemical analyzer, AU5800 (Beckman Coulter, Inc., Brea, CA, USA).

Fixed kidney tissues were prepared for 4-μm-thick paraffin-embedded sections. The hematoxylin–eosin (H&E) staining method was used to evaluate the tubular cell injury as described previously [15]. The percentage of injured tubules that displayed cellular necrosis, dilatation, cell swelling and loss of brush border were counted and scored as follows: 0, none; 1, 0-10%; 2, 11–25%; 3, 26–45%; 4, 46–75%; and 5, >75%. At least 10 randomly chosen high-powered fields (HPFs, × 400 magnification) for each sample were evaluated in a blinded manner, and an average score was calculated.

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay

Tubular cell death after IRI was assessed using an *in situ* cell death detection kit, (TMR red, Cat. No: 12156792910, Roche Diagnostics, Mannheim, Germany) as previously described [16]. Nuclei were visualized by a counterstaining with 4′,6-diamidino-2-phenylindole (DAPI, Cat. No: 10236276001, Roche Diagnostics, Mannheim, Germany). TUNEL-positive nuclei were identified by fluorescence microscopy and counted using

ImageJ analysis software 1.44 C (National Institutes of Health, MD, USA).

Cell culture and treatments

Immortalized human renal proximal tubule (HK-2) cells (American Type Culture Collection, Manassas, VA, USA) were cultured and subjected to the model of oxygen-glucose deprivation (OGD)/reoxygenation induced by incubating cells in a hypoxic environment for 90 min followed by 24 h of reoxygenation as described [15].

Adenovirus-mediated dgke gene overexpression

A premade adenovirus harboring an open read frame (ORF) of human *Dgke* with a C terminal Flag and His tag was purchased from Vigene Biosciences (Cat. No: VH876959, Rockville, USA). HK-2 cells were infected with adenovirus 12 h before OGD treatment at a multiplicity of infection (MOI) of 20 in the medium without antibiotics according to the manufacturer's instructions.

Klf15 gene silencing

A pool of three independent small interfering RNAs (siRNAs) targeting to different DNA sequences of human *Klf15* (accession number: NP_054798) were used to achieve strong on-target knockdown efficiency (GenePharma Co., Ltd, Shanghai, China). The sequences of the siRNA oligonucleotides used in this study were as follows: No 1: 5′-CCAGUGGACGAGAACUUCUTT-3′, No 2: 5′-GGAGGAGAUUGAAGAGUUUTT-3′, and No 3: 5′-CC UCCAAGUUUGUGCGCAUTT-3′. Both siRNA for the target gene and equivalent scramble control were delivered into cells using the Lipofectamine 2000 reagent (Cat. No: 11668-027, Invitrogen, Carlsbad, USA) according to the manufacturer's instructions.

Recombinant klotho protein treatment

HK-2 cells were incubated with recombinant human Klotho protein (rKlotho, Cat. No. ab84027, Abcam, Cambridge, USA) or vehicle (PBS) 6 h prior to OGD with a final concentration of 50 ng/mL in the medium.

Flow cytometry

Cell death after OGD/reoxygenation was determined by fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) staining as described [17].

RNA extraction and real-time RT-PCR

Total RNA was extracted from the kidney tissues or cells with TRIzol Reagent (Cat. No: 15596018, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The mRNA levels of target genes were analyzed by real-time quantitative RT-PCR using an iQ real-time PCR system (Bio-Rad, Hercules, CA, USA) as described previously [18]. Levels of the housekeeping gene were used as an internal control for the normalization of RNA quantity and quality differences among the samples. The primers for the target genes in this study are listed in Table 1.

Western blot analysis

Total protein from kidney tissue or cell pellets was extracted with RIPA lysis buffer (Cat. No: P0013B, Beyotime Biotechnology, Shanghai, China) containing 100 nM PMSF (Cat. No: 93482, Sigma-Aldich, St. Louis, MO, USA) and 1% protein inhibitor cocktail (Cat. No: PIC 0005, Sigma-Aldich) on ice followed by separation with SDS-PAGE. The samples were then transferred to PVDF membranes and incubated with antibodies. The antibodies used in this study are summarized in Table 2.

Table 1. Primer pairs of target genes used for real-time RT-PCR in this study.

Genes	Accession No.	Forward	Reverse
Dgke (mouse)	XM_011249151.4	TGGTCCTATGGACGCTGTG	CTGAACAGGTCGGTGTCACG
Dgke (human)	NM_003647.3	GACGGGCACCTGATCTTGTG	CTGGAGGCTACACCAGAAGG
Dgke (rat)	XM_032914054.1	CTGGGGTACTGGCTATGCTG	GGGCATCGGGTCCAATAGAG
Klf15 (mouse)	XM_030255539.2	GAGACCTTCTCGTCACCGAAA	GCTGGAGACATCGCTGTCAT
Klotho (mouse)	NM_013823.2	CAGGGTGAATGGTATCTGAAT	CTGTCTGCTTGGCACTGTTAT
Mcp-1(mouse)	NM_011333.3	ACCTGCTGCTACTCATTCAC	TTGAGGTGGTTGTGGAAAAG
Mcp-1(human)	NM_002982.3	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT
Il-6 (mouse)	NM_031168.1	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
Il-6 (human)	XM_005249745.2	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
Tnf-α (mouse)	NM_001278601.1	GAAAAGCAAGCAGCCAACCA	CGGATCATGCTTTCTGTGCTC
Tnf-α (human)	NM_000594.3	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
Gapdh (human, rat, mouse)	NC_000012.12(human) NM_017008.4 (rat) NM_008084.2 (mouse)	TGCATCCTGCACCACCAACTGC	ACAGCCTTGGCAGCACCAGTGG
β-actin (mouse)	NM_007393.3	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
β-actin (human)	XM_006715764.1	GAAGTGTGACGTGGACATCC	CCGATCCACACGGAGTACTT

Table 2. Primary antibodies used in this study.

Primary antibodies	Host	Dilution and supplier	Catalog number	Application
DGKE	Mouse	1:100, Santa Cruz, Dallas, USA	sc-100372	WB
DGKE	Rabbit	1:50, Abcam, Cambridge, USA	ab239024	IHC, IF
DGKE	Mouse	1:1000, R&D system, Minneapolis, USA	MAB5125	WB
KIM-1	Rabbit	1:100, Abcam, Cambridge, USA	ab216792	IF
CD68	Mouse	1:100, AbD Serotec, Oxiford, UK	MCA1957	IHC
Ly6B	Rat	1:100, Bio-Rad, Hercules, USA	MCA771	IHC
Klotho	Rabbit	1:1000 (WB), 1:100 (IHC), Abcam, Cambridge, USA	ab181373	WB, IHC
KLF15	Mouse	1:1000 (WB), 1:100 (IHC), Santa Cruz, Dallas, USA	sc-271675	WB, IHC
AQP-1	Goat	1:100, Abcam, Cambridge, USA	ab68387	IF
Calbindin D28K	mouse	1:100, Santa Cruz, Dallas, USA	sc-365360	IF
AQP3	mouse	1:100, Abcam, Cambridge, USA	sc-518001	IF
GAPDH	Rabbit	1:10000, Abways Technology, Shanghai, China	AB0037	WB

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescent staining methods were performed as previously described [19]. Negative controls using isotype matched normal IgG were performed to assess for antibody specificity. Images were obtained by an LSM780 laser-scanning confocal microscope equipped with a Plan-Apochromat at 63x/1.4 objective (ZEISS, Oberkochen, Germany). The total integrated density (IOD) for sections from different groups was analyzed by using the ImageJ analysis software (NIH). To define the tubular segment-specificity of DGKE expression in the kidney, double immunofluorescent staining was utilized for the identification of DGKE and various tubular cells in the kidney by using segment-specific cell markers based on previous studies [14]. Information on the antibodies is summarized in Table 2.

Statistics

Data are expressed as the mean ± SEM. All data were obtained from at least three independent experiments. Statistical analysis was performed in GraphPad Prism (version 8.0, GraphPad Software, San Diego, CA, USA). Comparisons between two groups were performed using two-tailed Student's t test. Differences between multiple groups with one variable or more than one variable were determined by one-way ANOVA and two-way ANOVA respectively. $p < 0.05$ was considered statistically significant.

Results

DGKE expression was time-dependently altered in the kidneys from mice with renal IRI and patients with biopsy-proven acute tubular necrosis

We first characterized the expression profile of DGKE in different tissues including adult murine brain, testis, heart, liver, spleen, lung, kidney, stomach, intestine and skeletal muscle by RT-PCR and Western blot analyses. DGKE was present in all these tissues with relatively high expression in the brain, testis and kidney (Figure 1(a and b)). In the kidney, DGKE was expressed in various types of renal parenchymal cells, such as human renal proximal tubule (HK-2) cells and podocytes (HPC), rat proximal tubule epithelial cells (NRK-52E), rat glomerular mesangial cells (RMC) and glomerular endothelial cells (GENC) as shown in Figure 1(c and d).

In ischemic mice, DGKE levels were time-dependently changed in the kidney after 30 min of ischemia followed by different time points of reperfusion as assessed by real-time RT-PCR (Figure 1e), Western blot (Figure 1(f)) and immunohistochemical staining analyses (Figure 1(g)). DGKE levels increased and peaked at 24 h after reperfusion. These values were subsequently reduced even lower than the basal level at 48 h or 72 h after reperfusion. Moreover, compared with kidney tissues obtained from patients who underwent tumor nephrectomies without renal disease, DGKE downregulation was also observed in paraffin embedded sections from the patients with biopsy-proven ATN accompanied by ischemic pathological manifestations or after renal transplantation (Figure 1(h)). To further define the tubular segment-specificity of DGKE expression in the kidney, double immunofluorescence staining showed that DGKE was mainly expressed in proximal convoluted tubules and distal convoluted tubules (Figure 1(i)).

DGKE overexpression ameliorated renal IRI in mice

Mice overexpressing DGKE (*Rosa26-Dgke*$^{+/+}$) were generated based on a technique wherein the target proteins were expressed under the ubiquitous *Rosa26* transcriptional machinery [20], a significant increase in DGKE expression was noted in the kidney based on real-time RT-PCR (Figure 2(a)) and Western blot analysis (Figure 2(b)). *Rosa26-Dgke*$^{+/+}$ mice were phenotypically

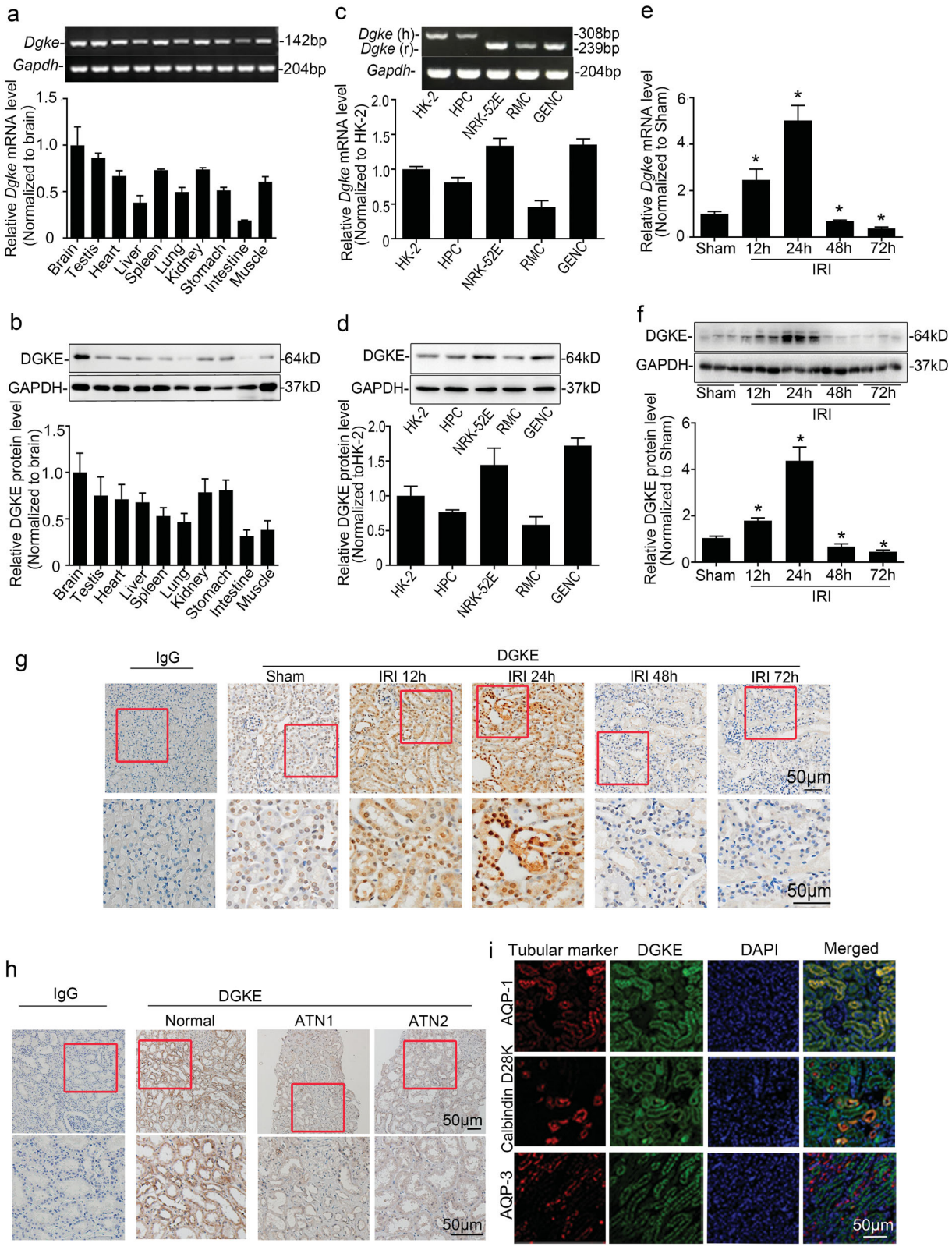

Figure 1. DGKE expression was time-dependently altered in the kidneys from mice with renal ischemia/reperfusion injury and patients with biopsy-proven acute tubular necrosis. (a) RT-PCR analysis of *Dgke* mRNA levels in selected murine tissues, including brain, testis, heart, liver, spleen, lung, kidney, stomach, intestine and skeletal muscle. (b) Western blot analysis of relative protein levels of DGKE in selected murine tissues. (c) RT-PCR analysis of *Dgke* mRNA levels in renal cells, including human renal proximal tubule (HK-2) cells and podocytes (HPC), rat proximal tubule epithelial cells (NRK-52E), rat glomerular mesangial cells (RMC) and glomerular endothelial cells (GENC). (d) Western blot analysis of the relative DGKE protein levels in renal cells. (e) Relative mRNA levels of *Dgke* in the mice kidneys after renal ischemia/reperfusion injury (IRI). (f) Representative Western blot gel documents and

Figure 1. (Continued).

summarized data showing DGKE protein levels in the mice kidneys after renal IRI. (g) Representative photomicrographs of 3, 3′-diaminobenzidine (DAB) immunohistochemical staining of DGKE in the mice kidneys after renal IRI. (h) Representative photomicrographs of DGKE immunohistochemical staining in the kidneys of patients with biopsy-proven acute tubular necrosis (ATN1 and ATN2 represent photomicrographs from patients with ATN accompanied by ischemic pathological manifestations or after renal transplantation, respectively). Normal kidney tissues were obtained from the healthy kidney poles of individuals who underwent tumor nephrectomies without renal disease. (i) Representative coimmunofluorescence staining sections following segment-specific tubular markers: proximal tubule, aquaporin-1 (AQP-1); distal tubule, calbindin D28k; and collecting duct, aquaporin-3 (AQP-3). It was found that DGKE was expressed in both proximal tubules and distal tubules. * $P < 0.05$ versus sham-operated mice (Sham) (n = 8).

normal and had no appreciable difference in renal morphology and function compared with WT mice. However, DGKE overexpression significantly improved renal functions, as shown by lower serum creatinine (Figure 2(c)) and blood urea concentrations (Figure 2(d)) in mice with renal IRI. WT mice with renal IRI showed obvious tubular damage that presented as loss of the brush border, tubular dilation, cast formation and tubular cell necrosis, whereas the DGKE overexpression group had less severe morphological manifestations, as shown in Figure 2(e). Consistently, DGKE overexpression in mice markedly reduced cell death according to the TUNEL assay results (Figure 2(f)) and the expression level of KIM-1 (Figure 2(g)), a reliable biological marker for the detection of proximal tubule damage following IRI [21].

DGKE overexpression alleviated inflammatory responses in the kidneys of mice with IRI

As shown in Figure 3(a), we further found that renal ischemia/reperfusion markedly enhanced the mRNA levels of proinflammatory mediators, including *Mcp-1*, *Il-6* and *Tnf-α,* and the infiltration of inflammatory cells, including neutrophils (Figure 3(b)) and macrophages (Figure 3(c)). These effects were attenuated by DGKE overexpression in mice.

DGKE overexpression recovered the expression levels of klotho and Krüppel-like factor 15 in the kidneys of mice with IRI

We found that Klotho mRNA and protein levels were significantly reduced in the kidney after 30 min of ischemia followed by 48 h of reperfusion, which were partly reversed by DGKE overexpression (Figure 4(a and b)). A similar tendency was found for KLF15 expression levels (Figure 4(c and d)). These results were further confirmed in paraffin-embedded sections of kidney tissues by immunohistochemical staining (Figure 4(e and f)).

KLF15-mediated klotho signaling was associated with the protective effect of DGKE in HK-2 cells under hypoxic conditions

To elucidate the interaction among DGKE, Klotho and KLF15, cultured HK-2 cells were subjected to OGD/reoxygenation *in vitro*. In consistent with *in vivo* studies, DGKE overexpression by adenoviral plasmid transfection (Figure 5(a)) significantly reduced hypoxia-induced tubular cell death and the production of proinflammatory mediators (Figure 5(b and c)). Furthermore, DGKE overexpression recovered the expression levels of Klotho and KLF15, which were reduced by hypoxia (Figure 5(d)). Gene silencing of *Klf15* (Figure 5(e)) counteracted the effects of DGKE on cell death (Figure 5(f)) and the expression of Klotho (Figure 5(g)). On the other hand, under hypoxic conditions, DGKE (Figure 5(h)) and KLF15 (Figure 5(i)) expression levels were not significantly changed in HK-2 cells pretreated with recombinant Klotho protein, indicating that Klotho might be a downstream target of DGKE and KLF15. Collectively, these results indicate that KLF15 is a bridge linking DGKE and Klotho signaling.

Discussion

Clinical studies have reported that loss-of-function mutations of DGKE cause a group of rare renal diseases called DGKE nephropathy. Although emerging findings further highlight the endothelial cell injury and podocyte dysfunction in DGKE nephropathy [7, 10, 22], the function of DGKE in AKI remains unknown. Therefore, the present study was designed to explore the potential role of DGKE in AKI induced by ischemia. In this study, one of the most important findings was a significant reduction in DGKE in human kidney sections from subjects with ischemia-associated acute tubular necrosis (ATN), which presents with AKI and is one of the most common causes of AKI, indicating the involvement of DGKE in the pathogenesis of AKI. Furthermore, by generating mice with DGKE overexpression, we confirmed the protective role of DGKE in renal IRI. Meanwhile, we also found that DGKE expression levels

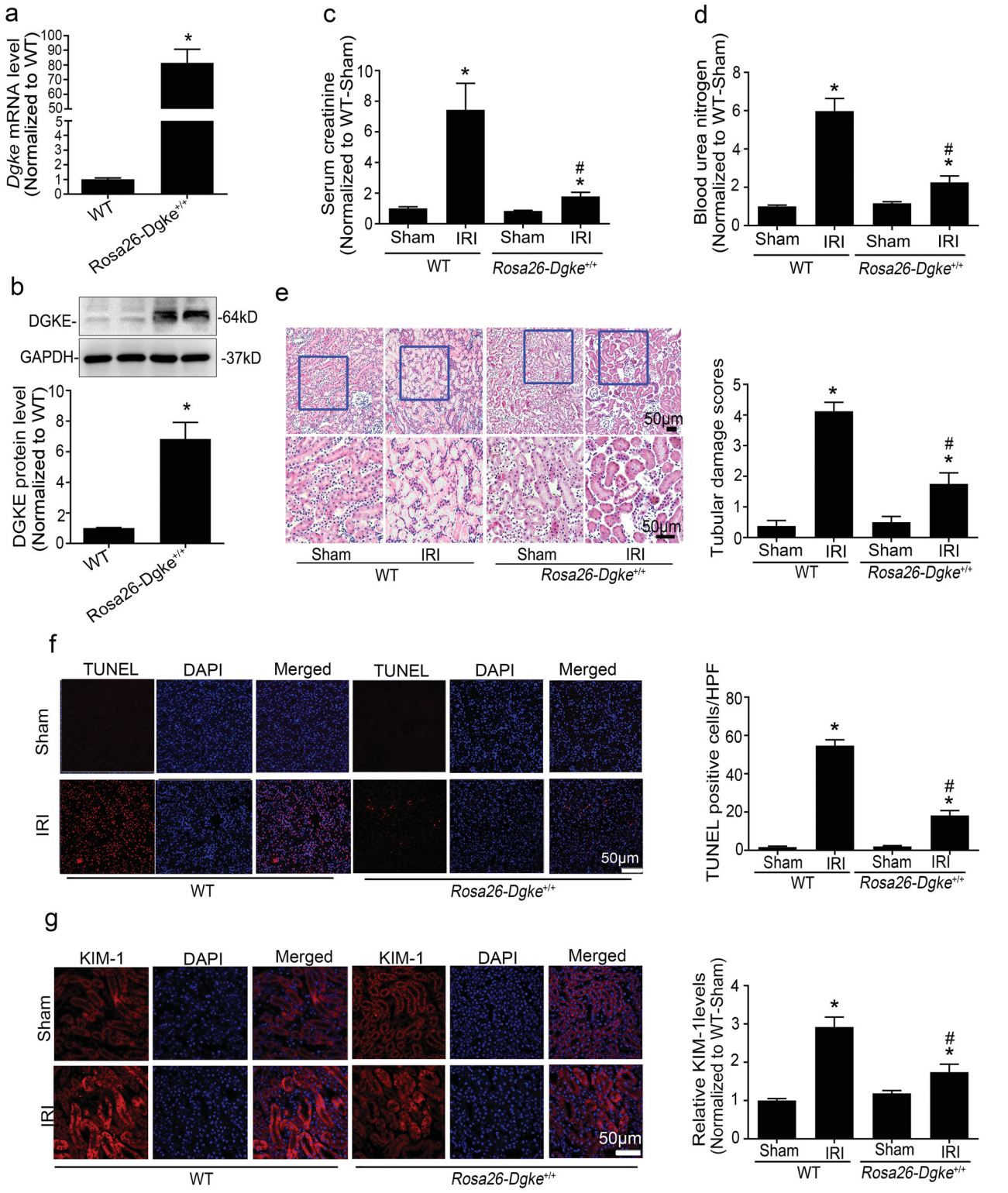

Figure 2. DGKE overexpression ameliorated renal ischemia/reperfusion injury in mice. (a) Relative mRNA levels of *Dgke* in the kidneys of WT and *Rosa26-Dgke*$^{+/+}$ mice. (b) Representative Western blot gel documents and summarized data showing the protein levels of DGKE in the kidneys of WT and *Rosa26-Dgke*$^{+/+}$ mice. (c) Serum creatinine concentration in different groups of mice. (d) Blood urea nitrogen levels in different groups of mice. (e) Representative micrographs showing the morphology based on hematoxylin and eosin (H&E) staining and quantitative assessment of tubular damage of the kidneys from different groups of mice. (f) *In situ* terminal deoxynucleotidyl transferase–mediated UTP nickend labeling (TUNEL, red) assay and quantitative assessment of tubular cell death (numbers per high-power field [HPF]. Nuclei were revealed using 4′, 6-diamidino-2-phenylindole (DAPI, blue) staining. (g) Representative immunofluorescence staining sections and quantitative analysis of KIM-1 expression levels in the kidneys from different groups of mice. $^{*}P < 0.05$ versus sham-operated wild-type mice (WT-Sham); $^{\#}$ $P < 0.05$ versus ischemic wild-type mice (WT-IRI) at the same experimental conditions (n = 8).

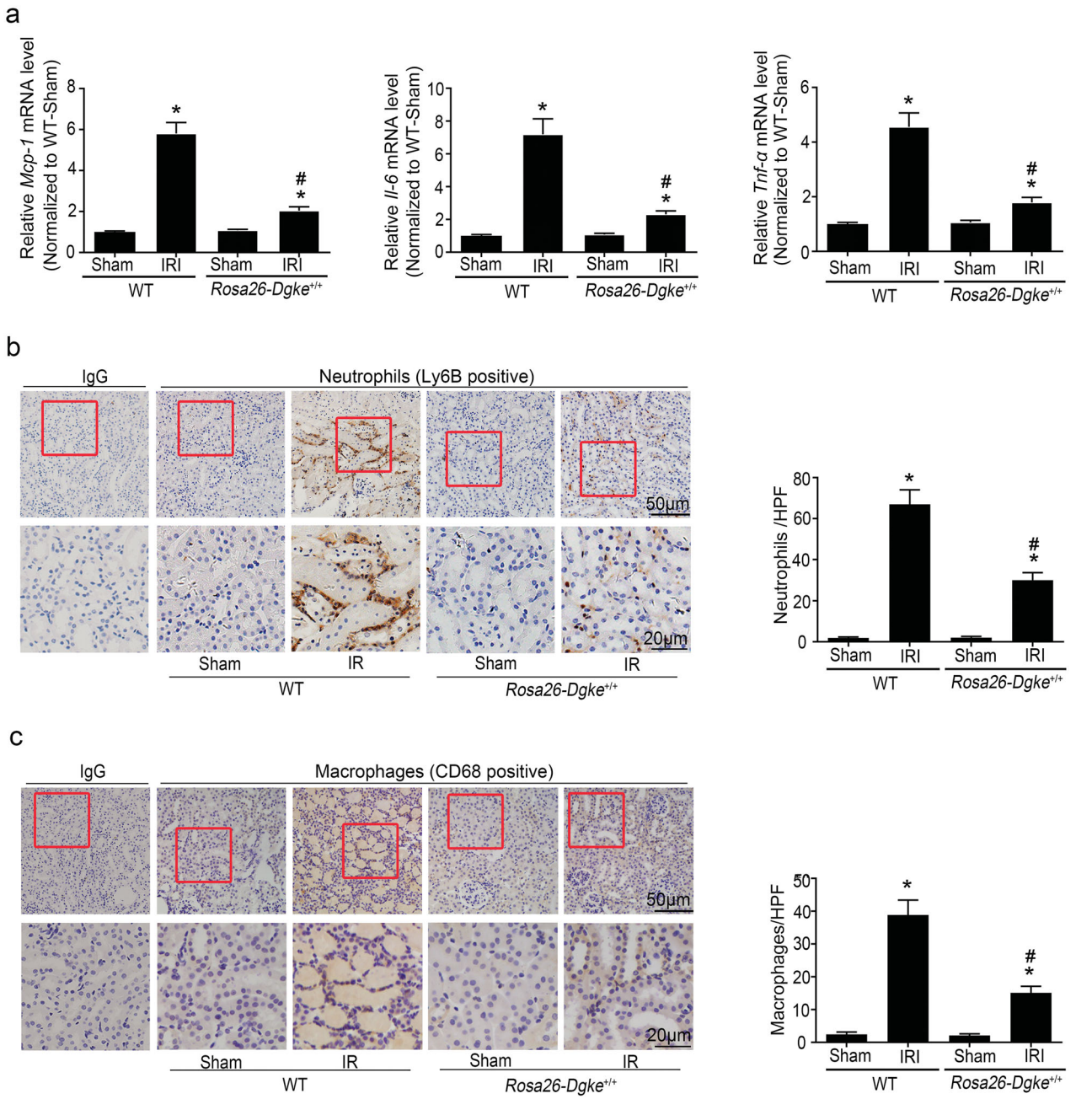

Figure 3. DGKE overexpression alleviated inflammatory responses in the kidneys of mice with renal ischemia/reperfusion injury. (a) The mRNA levels of proinflammatory mediators including *Mcp-1* (monocyte chemoattractant protein-1), *Il-6* (interleukin-6) and *Tnf-α* (tumor necrosis factor-α) were measured by real-time RT-PCR analysis. (b) Representative photomicrographs and analysis data for neutrophil (Ly6B positive) infiltration in the renal interstitium from different groups of mice. (c) Representative photomicrographs and analysis data for macrophage (CD68 positive) infiltration in the renal interstitium from different groups of mice. * $P < 0.05$ versus sham-operated wild-type mice (WT-Sham); # $P < 0.05$ versus ischemic wild-type mice (WT-IRI) under the same experimental conditions (n = 8).

were time-dependently changed in the kidneys from mice with renal IRI. It should be noted that the level of DGKE increased and peaked at 24 h after reperfusion. Then, these levels were markedly reduced to lower than the basal level at 48 h or 72 h after reperfusion in a time-dependent manner. On the basis of these observations, we speculated that the induction of DGKE expression at the early stage during renal IRI may serve as a compensatory strategy but does not sufficiently to suppress tubular damage.

Mechanistically, DGKE protects against AKI in mice, at least in part through Krüppel-like factor 15/Klotho signaling pathway. Based on microarray analysis of global gene expression in the kidney from mice with renal IRI in our previous studies (Microarray datasets were deposited to Gene Expression Omnibus under

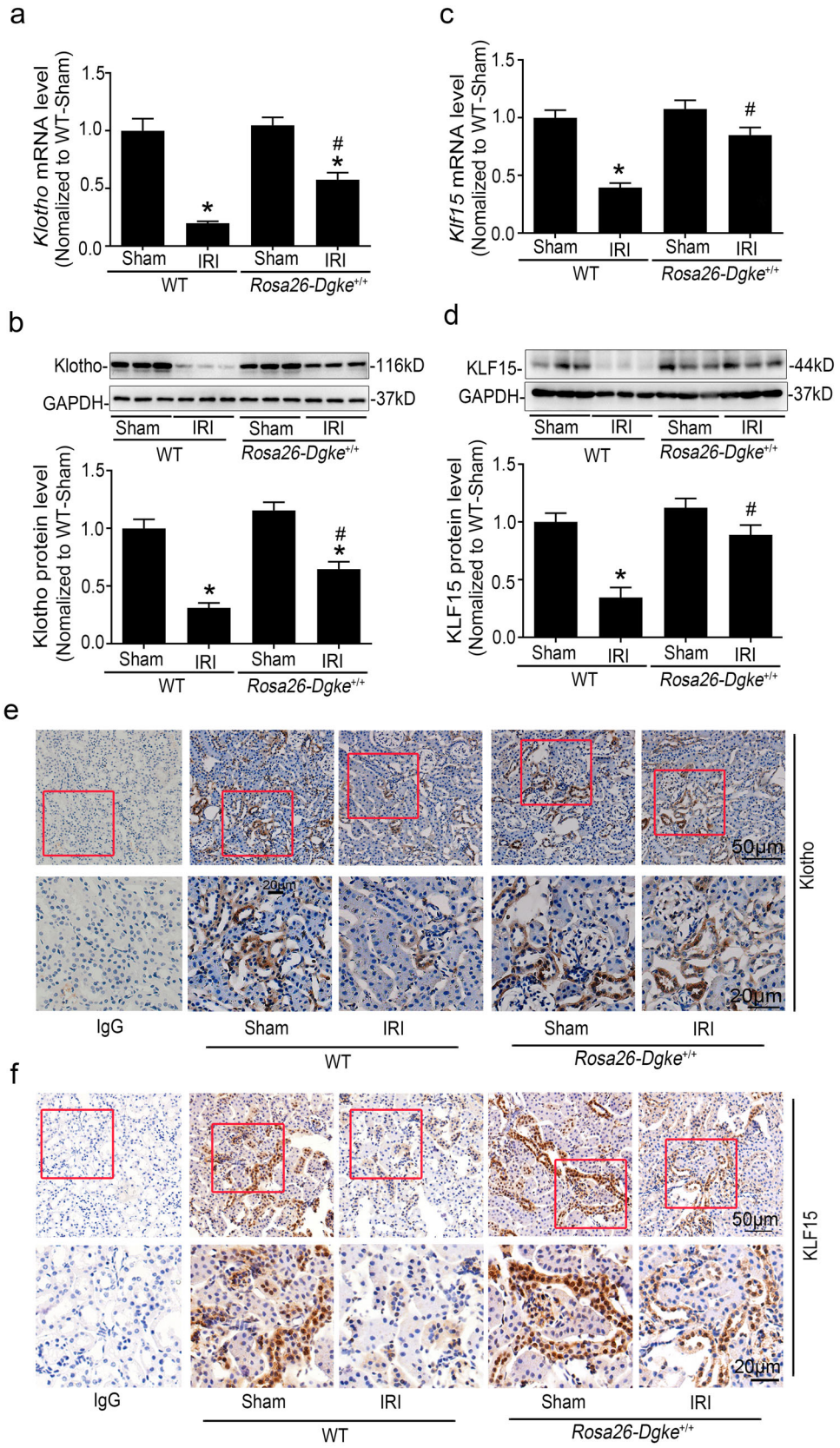

Figure 4. DGKE overexpression recovered Klotho and KLF15 expression levels in kidneys from mice with renal ischemia/reperfusion injury. (a) Relative *Klotho* mRNA levels in the kidney from different groups of mice. (b) Relative Klotho protein levels in kidneys from mice with IRI. (c) Relative Krüppel-like factor (*Klf*)15 mRNA levels in kidneys from different groups of mice. (d) Relative KLF15 protein levels in the kidneys from mice with IRI. (e) Representative immunohistochemical staining photomicrographs of Klotho in the kidney sections from different groups of mice. (f) Representative immunohistochemical staining photomicrographs of KLF15 in the kidney sections. * $P < 0.05$ versus sham-operated wild-type mice (WT-Sham); # $P < 0.05$ versus ischemic wild-type mice (WT-IRI) at the same experimental conditions (n = 8).

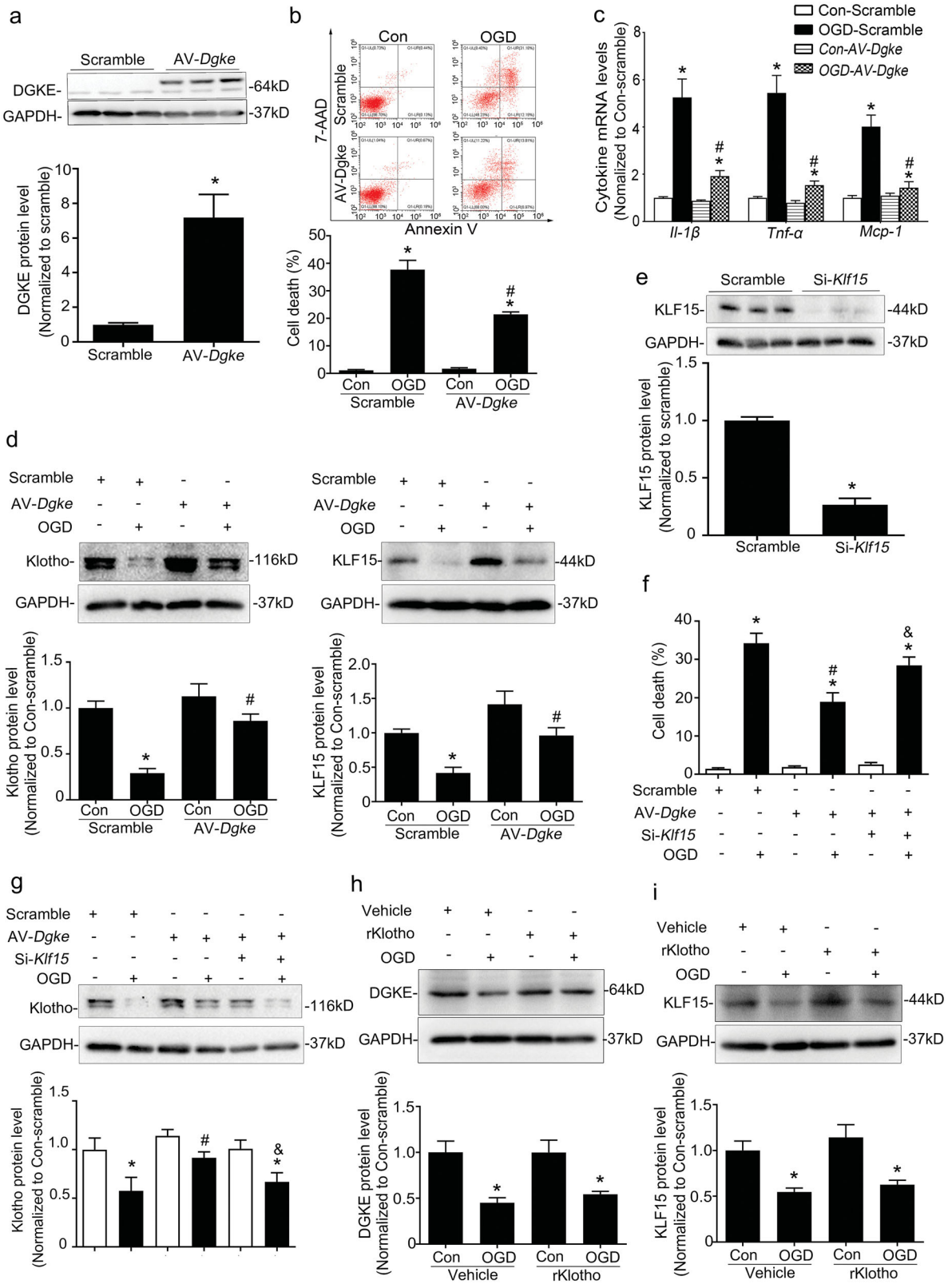

Figure 5. KLF15-mediated Klotho signaling was associated with the protective effect of DGKE in HK-2 cells under hypoxic conditions. (a) Representative Western blot gel documents and summarized data showing *Dgke* overexpression by adenovirus (AV) infection. (b) Summarized data showing the overall percentage of cell death, including the amount of apoptotic and necrotic cells determined by flow cytometric analysis in HK-2 cells under oxygen-glucose deprivation (OGD)/reoxygenation conditions. (c) The effect of DGKE on the mRNA levels of proinflammatory mediators in HK-2 cells under hypoxic conditions. (d) Representative Western blot results and summarized data showing the effect of DGKE on Klotho and KLF15 expression in HK-2 cells under OGD/reoxygenation conditions. (e) The gene silencing efficiency of siRNA targeting *Klf15*. (f) The percentage of HK-2 cell death with

Figure 5. (Continued).

different treatments. (g) Summarized data showing the levels of Klotho in HK-2 cells with different treatments. (h) Summarized data showing the DGKE level in HK-2 cells treated with recombinant Klotho (rKlotho) or vehicle. (i) The KLF15 level in HK-2 cells treated with rKlotho or vehicle. * $P < 0.05$ versus cells transfected with scramble sequence or vehicle treatment under normal conditions (Con-scramble/vehicle); # $P < 0.05$ versus cells transfected with scramble sequence under OGD conditions (OGD-scramble); & $P < 0.05$ versus cells transfected with adenovirus-*Dgke* under OGD condition (OGD-AV-*Dgke*) (n = 6).

accession code GSE192532), we found that some important key regulators, including KLF15 and Klotho, were significantly reduced in the kidney from ischemic mice. Meanwhile, using mass spectrum analysis, we also found that DGKE protein in podocytes can bind to KLF15, indicating a potential interaction between DGKE and KLF15 (unpublished data). It is known that KLF15 is a member of the KLFs, which are a group of zinc-finger DNA-binding transcription factors involved in various biological processes, including cell differentiation, metabolism, inflammation, apoptosis, mitochondrial biogenesis and DNA repair [23]. Emerging evidence has demonstrated that KLFs also regulate key physiological and pathological processes in the kidney [24], some of them have been considered as early diagnosis biomarkers or therapeutic targets in AKI [25] and chronic kidney diseases [26]. Among the KLF family members, KLF15 is widely distributed in the glomeruli and in the proximal tubule. It has been known that KLF15 plays a critical role in the kidney and is involved in tubular physiology, podocyte injury and renal fibrosis [27]. In particular, a recent study showed that KLF15 was significantly reduced in proximal tubule cells after aristolochic acid I (AAI) treatment, a proximal tubule-specific injury model. Proximal tubule specific knockout of KLF15 exacerbated proximal tubule injury and kidney function decline compared to control mice [28]. Based on these studies, we assessed whether the protective effect of DGKE is associated with KLF15. In this study, we found that DGKE overexpression recovered KLF15 expression levels in the kidneys of mice with IRI. In *in vitro* studies, we further found that *Klf15* gene silencing abolished the protective effect of DGKE on hypoxia-induced HK-2 cell injury. Taken together, our studies suggest that DGKE ameliorates renal IRI possibly through KLF15 signaling pathways.

Moreover, we also found that DGKE regulated the expression of Klotho, which was initially identified as an antiaging protein and is also mainly expressed in the kidney [29]. Numerous studies have indicated that Klotho is significantly correlated with the development and progression of AKI and CKD. Exogenous supplementation or overexpression of endogenous Klotho prevents and ameliorates injury, promotes recovery, and suppresses fibrosis to mitigate the development of CKD [30,31]. Therefore, Klotho is considered as a potential diagnostic biomarker and therapeutic target for the prevention of kidney injury [32]. However, the endogenous regulation of Klotho expression, release, and metabolism remains largely unknown. Considering that KLF15 is an important transcription factor involved in AKI, and that both Klotho and KLF15 are regulated by DGKE in this study, we therefore examined the interaction between Klotho and KLF15. Our results showed that *Klf15* gene silencing counteracted the effects of DGKE on Klotho expression. However, pretreatment with exogenous recombinant Klotho protein had no effects on KLF15 and DGKE expression, indicating that Klotho might be a downstream target of KLF15 directly or indirectly. Therefore, although our current data are very limited, we proposed that DGKE-induced Klotho expression is mediated, at least in part, by KLF15. Further studies are needed to detect whether Klotho expression is directly regulated by KLF15.

Conclusion

In summary, our studies demonstrate for the first time that DGKE is expressed in renal tubules and protects against renal ischemia/reperfusion injury, possibly through Krüppel-like factor 15/Klotho signal pathway, thereby contributing to alleviating inflammatory responses and tubular injury. A better understanding of the function of DGKE will provide unexpected opportunities for the development of new therapies for various renal diseases.

Ethical approval

The investigations of human renal biopsy samples in this study were conducted ethically in accordance with the principles of the World Medical Association Declaration of Helsinki and were approved by the Research Ethics Committee of School of Basic Medical Sciences, Shandong University (Document No. ECSBMSSDU2018-1-050) after informed consent was obtained from the patients. For animal experiments, all experimental procedures were performed in agreement with the Institutional Animal Care and Use Committee (IACUC) of School of Basic Medical Sciences, Shandong University (Document No. ECSBMSSDU2018-2-088) and conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Disclosure statement

The authors have no conflicts of interest to declare.

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